1277698

# THE UNITED STATES OF AMERICA

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*January 25, 2005*

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**APPLICATION NUMBER:** *60/556,737*
**FILING DATE:** *March 26, 2004*
**RELATED PCT APPLICATION NUMBER:** *PCT/US04/43499*

Certified by

Jon W. Dudas

Under Secretary of Commerce
for Intellectual Property
and Director of the United States
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PTO/SB/16 (01-04)
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# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV 389269752 US



## INVENTOR(S)

| Given Name (first and middle [if any]) | Family Name or Surname | Residence (City and either State or Foreign Country) |
|---|---|---|
| Song-Hua | Ke | San Diego, CA |
| Vijay K. | Mahant | Murrieta, CA |

*Additional inventors are being named on the _____ separately numbered sheets attached hereto*

## TITLE OF THE INVENTION (500 characters max)

Multiplexed Nucleic Acid Analysis With Improved Specificity

*Direct all correspondence to:* **CORRESPONDENCE ADDRESS**

[X] Customer Number: 34284

*OR*

| | | | | | |
|---|---|---|---|---|---|
| [ ] Firm or Individual Name | | | | | |
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| Address | | | | | |
| City | | State | | Zip | |
| Country | | Telephone | | Fax | |

## ENCLOSED APPLICATION PARTS *(check all that apply)*

[X] Specification *Number of Pages* 9
[ ] Drawing(s) *Number of Sheets* _____
[ ] Application Data Sheet. See 37 CFR 1.76
[ ] CD(s), Number _____
[ ] Other (specify) _____

## METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

[X] Applicant claims small entity status. See 37 CFR 1.27.
[ ] A check or money order is enclosed to cover the filing fees.
[X] The Director is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number: 502191
[ ] Payment by credit card. Form PTO-2038 is attached.

FILING FEE Amount ($): 80.00

The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

[X] No.
[ ] Yes, the name of the U.S. Government agency and the Government contract number are: _____



*Respectfully submitted,*

SIGNATURE _____

TYPED or PRINTED NAME Martin Fessenmaier

TELEPHONE 714-641-5100

Date 03/26/04

REGISTRATION NO. 46697 *(If appropriate)*

Docket Number: 100788.0023PRO

***USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT***

PTO/SB/17 (10-03)
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# FEE TRANSMITTAL for FY 2004

*Effective 10/01/2003. Patent fees are subject to annual revision.*

[X] Applicant claims small entity status. See 37 CFR 1.27

**TOTAL AMOUNT OF PAYMENT** ($) 80.00

| *Complete if Known* | |
|---|---|
| Application Number | |
| Filing Date | March 26, 2004 |
| First Named Inventor | Song-Hua Ke |
| Examiner Name | |
| Art Unit | |
| Attorney Docket No. | 100788.0023PRO |

## METHOD OF PAYMENT *(check all that apply)*

[ ] Check [ ] Credit card [ ] Money Order [ ] Other [ ] None

[X] Deposit Account:

Deposit Account Number: 502191

Deposit Account Name: Rutan & Tucker

**The Director is authorized to:** *(check all that apply)*

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## FEE CALCULATION

### 1. BASIC FILING FEE

| Large Entity Fee Code | Large Entity Fee ($) | Small Entity Fee Code | Small Entity Fee ($) | Fee Description | Fee Paid |
|---|---|---|---|---|---|
| 1001 | 770 | 2001 | 385 | Utility filing fee | |
| 1002 | 340 | 2002 | 170 | Design filing fee | |
| 1003 | 530 | 2003 | 265 | Plant filing fee | |
| 1004 | 770 | 2004 | 385 | Reissue filing fee | |
| 1005 | 160 | 2005 | 80 | Provisional filing fee | 80.00 |

SUBTOTAL (1) ($) 80.00

### 2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

| | Extra Claims | | Fee from below | = | Fee Paid |
|---|---|---|---|---|---|
| Total Claims | | -20** = | | X | = |
| Independent Claims | | - 3** = | | X | = |
| Multiple Dependent | | | | | = |

| Large Entity Fee Code | Large Entity Fee ($) | Small Entity Fee Code | Small Entity Fee ($) | Fee Description |
|---|---|---|---|---|
| 1202 | 18 | 2202 | 9 | Claims in excess of 20 |
| 1201 | 86 | 2201 | 43 | Independent claims in excess of 3 |
| 1203 | 290 | 2203 | 145 | Multiple dependent claim, if not paid |
| 1204 | 86 | 2204 | 43 | ** Reissue independent claims over original patent |
| 1205 | 18 | 2205 | 9 | ** Reissue claims in excess of 20 and over original patent |

SUBTOTAL (2) ($)

**or number previously paid, if greater; For Reissues, see above

## FEE CALCULATION (continued)

### 3. ADDITIONAL FEES

| Large Entity Fee Code | Large Entity Fee ($) | Small Entity Fee Code | Small Entity Fee ($) | Fee Description | Fee Paid |
|---|---|---|---|---|---|
| 1051 | 130 | 2051 | 65 | Surcharge - late filing fee or oath | |
| 1052 | 50 | 2052 | 25 | Surcharge - late provisional filing fee or cover sheet | |
| 1053 | 130 | 1053 | 130 | Non-English specification | |
| 1812 | 2,520 | 1812 | 2,520 | For filing a request for *ex parte* reexamination | |
| 1804 | 920* | 1804 | 920* | Requesting publication of SIR prior to Examiner action | |
| 1805 | 1,840* | 1805 | 1,840* | Requesting publication of SIR after Examiner action | |
| 1251 | 110 | 2251 | 55 | Extension for reply within first month | |
| 1252 | 420 | 2252 | 210 | Extension for reply within second month | |
| 1253 | 950 | 2253 | 475 | Extension for reply within third month | |
| 1254 | 1,480 | 2254 | 740 | Extension for reply within fourth month | |
| 1255 | 2,010 | 2255 | 1,005 | Extension for reply within fifth month | |
| 1401 | 330 | 2401 | 165 | Notice of Appeal | |
| 1402 | 330 | 2402 | 165 | Filing brief in support of an appeal | |
| 1403 | 290 | 2403 | 145 | Request for oral hearing | |
| 1451 | 1,510 | 1451 | 1,510 | Petition to institute a public use proceeding | |
| 1452 | 110 | 2452 | 55 | Petition to revive - unavoidable | |
| 1453 | 1,330 | 2453 | 665 | Petition to revive - unintentional | |
| 1501 | 1,330 | 2501 | 665 | Utility issue fee (or reissue) | |
| 1502 | 480 | 2502 | 240 | Design issue fee | |
| 1503 | 640 | 2503 | 320 | Plant issue fee | |
| 1460 | 130 | 1460 | 130 | Petitions to the Commissioner | |
| 1807 | 50 | 1807 | 50 | Processing fee under 37 CFR 1.17(q) | |
| 1806 | 180 | 1806 | 180 | Submission of Information Disclosure Stmt | |
| 8021 | 40 | 8021 | 40 | Recording each patent assignment per property (times number of properties) | |
| 1809 | 770 | 2809 | 385 | Filing a submission after final rejection (37 CFR 1.129(a)) | |
| 1810 | 770 | 2810 | 385 | For each additional invention to be examined (37 CFR 1.129(b)) | |
| 1801 | 770 | 2801 | 385 | Request for Continued Examination (RCE) | |
| 1802 | 900 | 1802 | 900 | Request for expedited examination of a design application | |

Other fee (specify) _____

*Reduced by Basic Filing Fee Paid SUBTOTAL (3) ($)

## SUBMITTED BY

(Complete *(if applicable)*)

| | | | | | |
|---|---|---|---|---|---|
| Name (Print/Type) | Martin Fessenmaier | Registration No. (Attorney/Agent) | 46697 | Telephone | 714-641-5100 |
| Signature | | | | Date | March 26, 2004 |

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This collection of information is required by 37 CFR 1.17 and 1.27. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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# MULTIPLEXED NUCLEIC ACID ANALYSIS WITH IMPROVED SPECIFICITY

## Field of The Invention

Molecular diagnosis and devices therefor.

## Background of The Invention

Human papillomavirus (HPV) is now considered a major cause of cervical cancer, killing more than 200000 women around the world each year. The HPV virus is relatively common and more than 100 distinct types of HPV have been identified, some of which are considered "high-risk" for the development of cancer. Detection of such high-risk types of HPV has significant impact on diagnosis, prevention, treatment and management of cervical cancer in HPV-infected women.

To date, molecular HPV diagnosis relies on various formats of hybridization technology, including southern blot, dot blot, line blot, and *in situ* hybridization. For example, HybridCapture 2 from Digene is a nucleic acid hybridization microplate assay based on chemiluminescence for the qualitative detection, and differentiating low-risk from high risk groups. Other commercially available tests employ similar methods and may detect presence of various types of HPV in a patient sample. However, currently known diagnostic methods based on hybridization often lack specificity due to cross-hybridization. Cross-hybridization may result in a false positive signal due to closely related types of HPV (*e.g.*, where a target DNA has only a single or few mismatches to the probes being used). To overcome problems associated with cross-hybridization, a number of approaches have been developed and used such as stringent hybridization and washing, use of PNA, super G and C, universal base stretch, locked DNA, etc. However, most of such approaches have limitations in the capability to increase specificity and frequently increase cost or automation difficulties.

Hybridization specificity may be driven by control of the melting temperature. However, $T_m$-specific hybridization will frequently lead to false positive results due to sequence similarity of closely related sequence variations. For example, non-specific signals may be obtained where a first sequence variation increases a $T_m$ while a second sequence variation decreases the $T_m$. In

addition to the above approach, the specificity may be further improved by employing multiple probes (e.g., using a second or third labeled probe for differentiating HPV subtypes.

Therefore, while there are numerous methods for nucleic acid based testing for HPV and other pathogens are known in the art, all or almost all of them suffer from various problems, which are even more aggravated where such analysis is performed in a multiplex environment (*e.g.*, a biochip). Consequently, there is still a need to provide improved methods and compositions for molecular diagnostics.

**Detailed Description**

The inventors generally contemplate diagnostic methods that include a primer extension step in which hybridization specificity is significantly improved by selective choice of PCR oligos for generation of the amplicon and matching choice of primer extension oligo. It should be recognized that contemplated methods are applicable in a variety of diagnostic tests, and that the nature of the nucleic acid to be tested may vary considerably. Therefore, suitable tests include genotyping of biological specimen, SNP analysis, etc., and may start from DNA as well as from RNA (hn RNA, mRNA, or other). Particularly preferred methods are performed using microarray and/or non-microarray methodologies, and offer higher specificity and sensitivity compared to currently used methodologies.

Typically, most contemplated methods will include a first amplification step in which the target material is amplified in a single (e.g., a single tube PCR) or multiple tubes for a subsequent primer extension analysis step. However, it should be recognized that such first amplification may not be necessary in all cases and it is therefore contemplated that the first amplification may be omitted entirely so long as the primer extension criteria match the test nucleic acid.

*Amplicon Generation*

In an especially contemplated aspect of the inventive subject matter, PCR oligos are designed such that two criteria are met: First, the choice of the oligos is dictated by the position of the sequence variation that is to be detected (wherein the sequence variation is characteristic to a particular genotype). Therefore, both primers will flank the sequence variation such that the

amplicon and not the primers will include the sequence variation. Second, the primer sequences must be chosen such that a DNA product is generated in the PCR to which the primer extension oligo can bind at a predefined melting point. Furthermore, where possible, allele specific primer may be employed for generating the extension product.

*Primer Extension*

Again, two conditions must be met by the primer extension oligo: First, the primer extension oligo is selected such that the oligo only binds to the intended target, but not to a similar genotype sequence at the same experimental condition. In most preferred aspects, such selectivity is achieved via Tm control. Alternatively, or additionally, salinity and/or dielectric constant of the medium may be altered to modulate binding selectivity. Furthermore, agents that disrupt hydrogen bonding (chaotropic agents) may be added. Second, the primer extension oligo is further selected such that extension will only occur with proper 3'-end base pairing (*i.e.*, only a perfect match at the 3'-end of the extension primer will allow extension).

Therefore, where multiple sequence variation are to be tested, it should be recognized that the amplicon primers are chosen such that the amplicon provides a hybridization target having a Tm and sequence that is distinct from the Tm and sequence of a second hybridization target of a second amplicon. Particularly preferred Tm differences are at least 1 °C, more typically at least 2 °C, and even more typically at least 4°C, while the sequence difference between first and second extension oligo is at least 1 nucleotide, more preferably at least 2 nucleotides, and most preferably at least 4 nucleotides.

It is still further preferred that the primer extension oligo will include a unique sequence (preferably with little or no homology [less than 50%] to the amplicon and/or extension product) that is used to hybridize the primer (with or without extension) to a predetermined corresponding nucleic acid that is preferably at a predetermined position on the chip. Addition of such unique sequence to the 5'-end will allow rapid correlation of a primer or extension signal to a predetermined position on an array. Moreover, it is also preferred that the extension oligo will carry a quantifiable marker (*e.g.*, fluorescent, luminescent, absorbing, or otherwise labeled) that allows normalization of the extension signal.

Primers complying with these conditions have been found to overcome problems with methods relying exclusively on Tm-specific hybridization, which tend to produce false positive results due to sequence similarity. Contemplated methods were also found to overcome problems associated with methods relying exclusively on correct 3'-match, which often lead to false positive results due to the inability of the extension polymerase to proof proper hybridization conditions towards the 5'-end.

Sometimes, for a specific type of HPV, random sequence variation may occur within the detect region, especially at the region where the 3' terminal of the detection probe binds. This situation will prevent primer extension and cause false negative results. To overcome this phenomenon, a second probe is employed at a different location within the same amplified region. The chance of random sequence variation at two defined location at the same time will be a rare occurrence. In the event the first probe fails to detect the virus, the second probe thus increases the probability of detecting the virus and this will thus minimize false negative results.

Figure 1 below depicts an exemplary flowchart for contemplated methods:

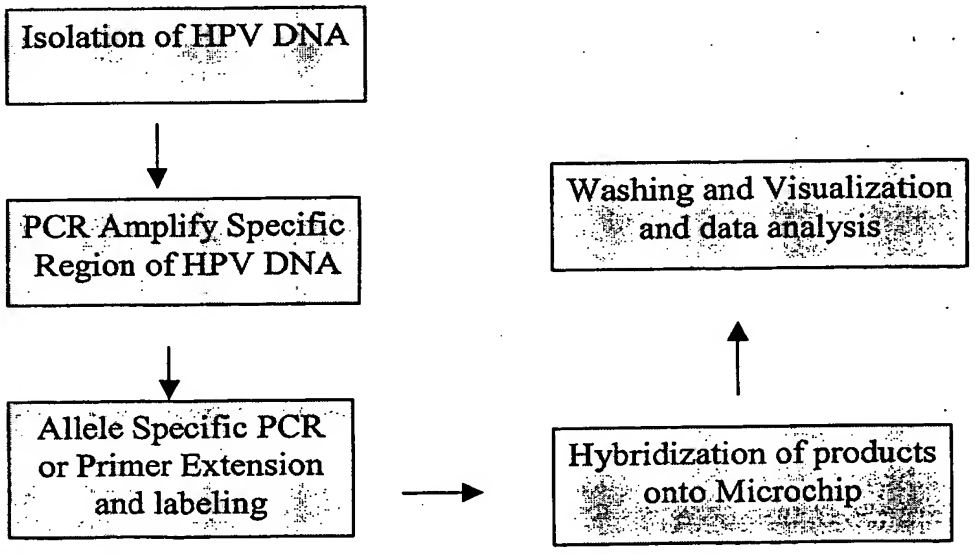
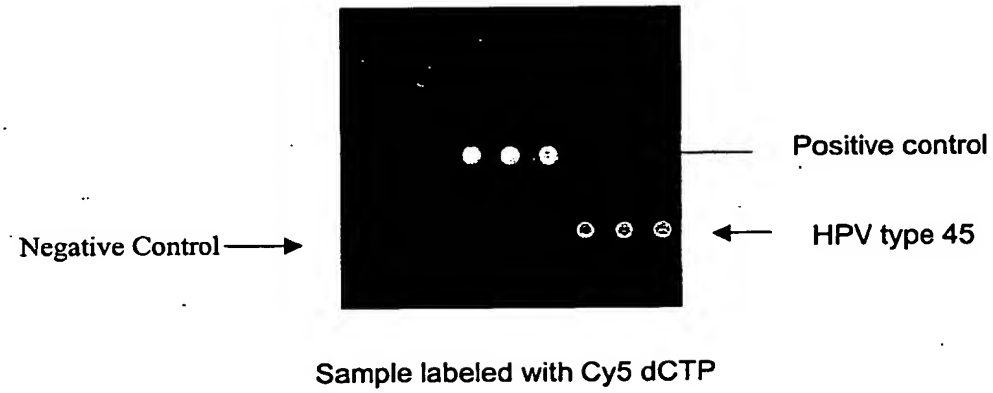



*Figure 1*

In a typical process according to Figure 1, DNA is isolated using a commercially available kit (*e.g.*, from QIAGEN or Gentra). The typing of HPV DNA is performed using allele specific PCR or primer extension. The subsequent procedure of hybridization of products onto chip is then performed to visualize or otherwise detect the typing results.

Figure 2 below provides an example of HPV detection from an actual patient sample. Here, each type of HPV probe is spotted in triplicate with positive and negative controls onto a biofilm chip. The result indicated HPV type 45 infection for the patient and the results were verified by DNA sequencing. Exemplary biofilm chips and devices are described in our copending applications with the serial numbers PCT/US02/03917, PCT/US01/47991, and 10/346879, which are incorporated by reference herein. Figure 3 (A) illustrates the detection results of HeLa cell infected with HPV 18 and Figure 3 (B) shows the results of Caski cell line infected with HPV 16. As in Figure 2, each type of HPV probe is spotted in triplicate with positive and negative control.

***Figure 2***



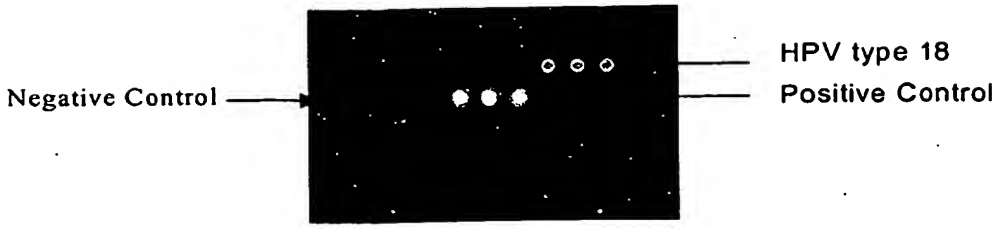



*Figure 3A*

A. Hela Cell HPV Detection

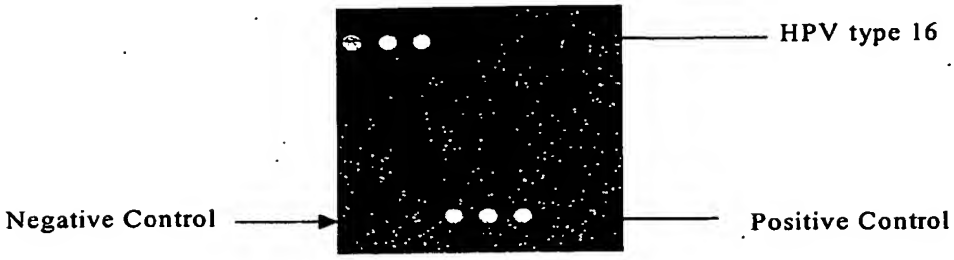



*Figure 3B*

B. Caski Cell HPV Detection

Figure 4 depicts the comparison results from the method according to the inventive subject matter (A) and from a conventional method based on hybridization (B) using the same DNA samples isolated from ME180 cells line. Figure 4 (A) indicates the presence of HPV-68 in the ME 180 cell line without ambiguous, but Figure 4 (B) shows positive signal for both HPV-29 and HPV-53 besides HPV-68. The extra erroneous signals for HPV-29 and HPV-53 were the results from cross-hybridization.

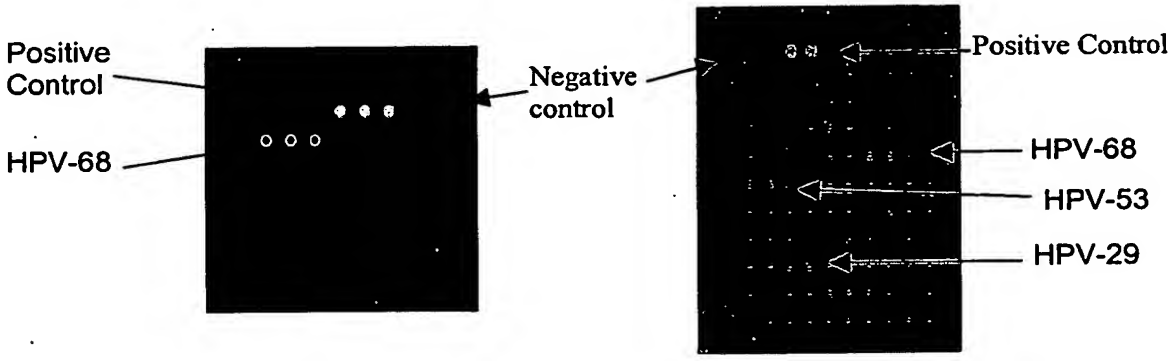



*Figure 4*

Thus, especially suitable methods may include the steps of (a) extraction of DNA from patient sample, (b) PCR amplification of a region of HPV genome, (c) allele specific asymmetric PCR or primer extension with primers specific to each type of HPV, and (d) optionally labeling the extension oligo. The preferred polymerase for primer extension is a polymerase capable of recognizing structure and conformational differences at or near the 3' end of a primer-template complex. In a typical detection, hybridization of the labeled primer extension products takes place with capture probes immobilized on a support (the label can be fluorescent, radioactive, chemiluminescent, phosphorescent, electrochemical, or chromogenic).

**Experimental Procedure**

Step 1: Isolate virus DNA from Pap Smear Samples using QIAamp DNA Blood Mini Kit Handbook from QIAGEN or using Genomic DNA Purification Kit from Gentra.

Step 2: Perform PCR to amplify target sequences in the region of E6 and E7 of HPV genome.

Step 3: Perform HPV Type-Specific Primer Extension Assay with Platinum GenoTYPE Tsp DNA Polymerase from Invitrogen. In this step, detectable fluorescent moieties (such as Cy5-dCTP or Alexa-dCTP) are incorporated into primer extension products.

Step 4: Apply sample onto Chips and perform hybridization with probes immobilized on the surface of the chips (HPV Genotyping Chips from AutoGenomics, Carlsbad, CA)

Step 5: Wash out unincorporated fluorescent moiety and un-hybridized primer extension product and detect signals with Cy3 or Cy5 scanning or other fluorescent detection systems.

*Amplicon Oligos*

Based on sequence homology at the DNA level, a set of primers or degenerated primers have been designed. The optimal primers have a Tm about 52 °C to 64 °C.

For HPV-16

Upstream Primer: 5' GTATATAGAGATGGGAATCC 3'

Downstream Primer: 5' GCCTCTACATAAAACCATCC 3'

For HPV-18

Upstream Primer: 5' GTGTATAGAGACAGTATACC 3'

Downstream Primer: 5' GCTTGTACATAAAACCAGCC 3'

*Primer Extension Oligos*

For probes of each HPV type, a unique sequences specific for each type of HPV are designed. These probe can be extended with Platinum GenoTYPE Tsp DNA polymerase (from Invitrogen) or other enzymes which can recognize the 3' end structure, only in the conditions that correct Watson-Crick base pairings are existing at the 3' end of detection probes and templates. The probes can be 8 to 40 bases in length with oligonucleotide tag sequences attached to them. The optimal probes have a Tm about 52 °C to 65 °C, but can be variable from 42 °C to 74 °C.

For HPV-16

Probe Sequence (ASPE): 5' GTTGCAGATCATCAAGAACAC 3'

*For HPV-18

Probe Sequence (ASPE) 5' CGACAGGAACGACTCCAACG 3'

It should be noted that contemplated compositions and methods invariably provided conclusive positive or negative results for a given HPV genotype, whereas some of alternative methods solely based on hybridization or primer extension would have provided inconclusive results (typically mistyping or false positive results). Furthermore, most results obtained using contemplated compositions and methods could be independently confirmed by running known assays in the art (which typically required multiple tests in at least some cases to obtain a conclusive result).

Thus, specific embodiments and applications of primer extension analyses with improved specificity have been disclosed. It should be apparent, however, to those skilled in the art that many more modifications besides those already described are possible without departing from the inventive concepts herein. The inventive subject matter, therefore, is not to be restricted except in the spirit of the present disclosure. Moreover, in interpreting the specification, all terms should be interpreted in the broadest possible manner consistent with the context. In particular, the terms "comprises" and "comprising" should be interpreted as referring to elements, components, or steps in a non-exclusive manner, indicating that the referenced elements, components, or steps may be present, or utilized, or combined with other elements, components, or steps that are not expressly referenced.

# Document made available under the Patent Cooperation Treaty (PCT)

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